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Review

Cancer stem cells with increased metastatic potential as a therapeutic target for esophageal cancer

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ABSTRACT

Esophageal cancers (EC) are highly aggressive tumors, commonly presented in a locally advanced stage with a poor prognosis and survival. Up to 50% of the patients are eligible for treatment with curative intent and receive the standard treatment with neoadjuvant chemoradiotherapy (nCRT) and surgery. Currently, pathologic complete response to nCRT is 20–30%, with a partial or no response in about 50% and 20%, respectively. EC recurrences occur frequently even after successful anti-cancer treatment, suggesting high aggressiveness with increased metastatic potential. A tumor sub-population of so-called cancer stem cells (CSCs), is known to display a high metastatic potential and resistance to conventional anti-cancer therapy, hereby being responsible for the unbeneficial clinical features. In this review, a concise overview will be given of the current literature on esophageal CSCs and related metastases. Esophageal CSC markers will be discussed followed by the pathways that initiate and sustain these cells. In addition, the cellular processes, epithelial-mesenchymal transition (EMT), hypoxia and autophagy, known to contribute to cancer stemness and metastasis will be explained. Finally, potential options for treatment also related to cancer genome atlas (TCGA) data on EC will be discussed.

1. Introduction

Esophageal cancer (EC) is currently the 8th most common malignancy worldwide and the 6th leading cause of cancer related death, accounting for more than 490 000 new cases and 400 000 deaths in 2014 (world cancer report 2014). The 5-year survival of this highly aggressive tumor is approximately 20% (www.cancer.org). At diagnosis, patients often present with locally advanced tumors, including lymph node involvement in more than 75%. Usually symptoms occur when the tumor has infiltrated over half of the circumference of the esophagus or has spread by direct local growth in the adventitial tissues, via lymph vessels to surrounding nodes and distantly through hematogenous dissemination. Distant metastases are frequently observed in the liver, lungs, bones, adrenal glands, kidney and brain [1]. There are two typical esophageal cancers, esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC). ESCC, predominantly present in the Eastern and Central Asian world, derives from dysplastic squamous cell epithelium that usually occurs in the upper two-third of the esophagus. EAC mainly develops in the distal esophagus, where ongoing gastroesophageal reflux esophagitis potentially transforms squamous epithelium into columnar intestinal epithelium that further evolves through low and high grade dysplasia into EAC [1,2]. Alcohol and tobacco are the most important risk factors of ESCC whereas EAC is associated with obesity, smoking and chronic gastroesophageal reflux disease (GERD) with premalignant Barrett's esophagus [3,4]. Nodal metastases occur frequently in the mid and upper mediastinum in ESCC and abdominal metastases in EAC [1]. The treatment of choice for locally advanced resectable tumors, both ESCC and EAC, is neoadjuvant chemoradiation (nCRT) followed by radical surgery [5]. Regretfully, around 20% of the tumors will not respond at all, more than 50% do not respond adequately, and even after pathologic complete response early and distant recurrences occur in most patients [6]. Therefore, it is necessary to investigate the sub-population of cells with increased treatment resistance and metastatic potential, the so-called cancer stem cells (CSCs) [7].

CSCs were first proposed by Virchow and Conheim; a subpopulation of cancer cells resembles the same traits as embryonic cells such as the ability to proliferate, and cancer is derived from the activation of dormant cells of the same tissue [8]. One of the first experiments confirming the existence of CSCs, showed indeed that only a limited percentage of transplanted primary tumor cells could initiate a secondary tumor [9]. Subsequent research used FACS and cell surface

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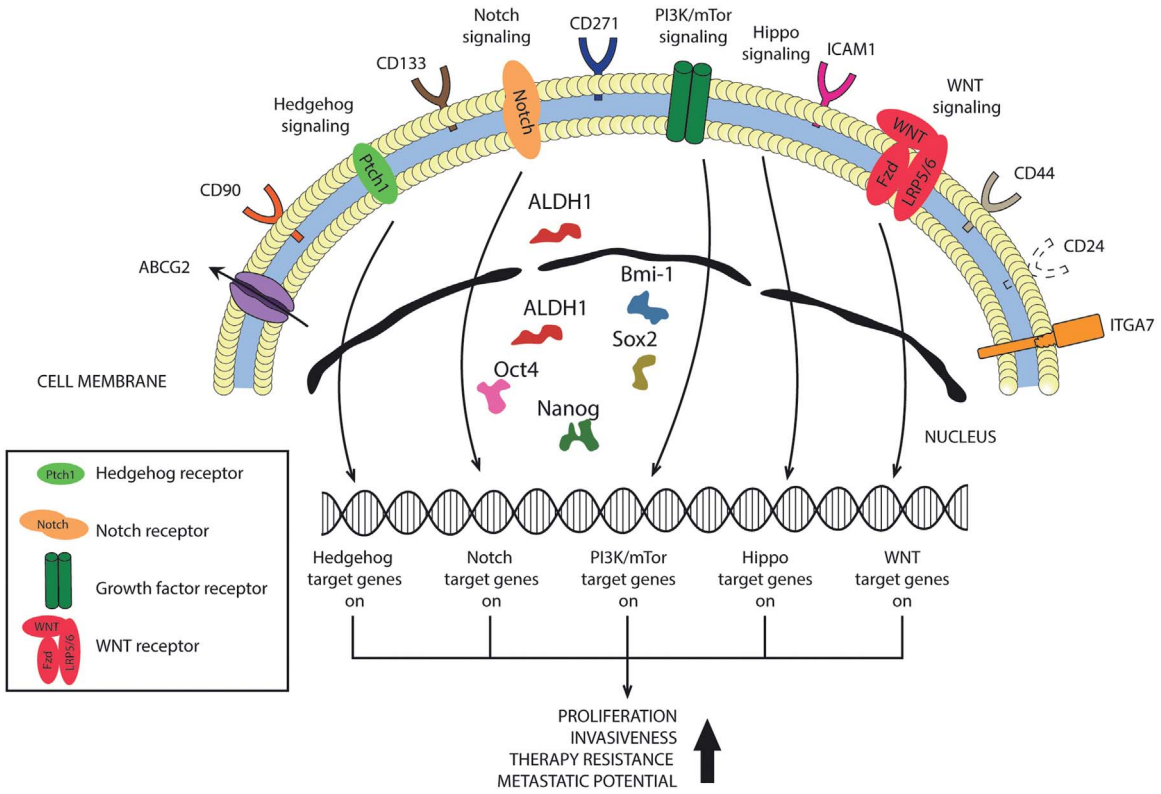

Fig. 1. Overview of markers and pathways defining esophageal CSC populations. Cell surface markers ABCG2, CD90, ITGA7 and CD44 are used as single markers while CD44 can be used in combination with CD24 ($CD44^+/CD24^-$), CD133 ($CD44^+/CD133^+$), the intracellular marker ALDH1 ($CD44^+/ALDH1^+$) and ICAM1 ($CD44^+/ICAM1^+$) to identify CSCs. ALDH1 can also be used as a single marker. CD133 can be used alone or in combination with ABCG2 and CXCR4. CD271 is another CSC marker. Other read-outs for cancer stemness are the transcription factors Bmi-1, Nanog, Sox2 and Oct4. Hedgehog, Notch, WNT, PI3K/mTOR and Hippo pathways are implicated to regulate CSC populations leading to more proliferation, invasiveness, therapy resistance and higher metastatic potential.

markers to further characterize CSCs and investigate mechanisms involved in the regulation of cancer stemness [10,11]. CSCs are, in contrast to non-CSCs, thought to be dormant or quiescent [12,13] and therefore therapy resistant but when re-entering the cell cycle are able to form recurrences or metastases [11,13,14]. In vitro cancer sphere forming potential and in vivo tumor initiating potential are often used as read-outs for cancer stemness [15–17]. It is believed that CSCs represent a small percentage of all EC cells with the majority part consist of more differentiated cells [18], albeit this has recently been challenged by studies showing plasticity of differentiated tumor cells [19,20].

This review will focus on EC CSCs as a target for eliminating resistant and highly metastatic cell populations and the role of tumor microenvironment in facilitating this process.

2. Markers to identify esophageal CSCs

Although the use of markers to select CSC enriched populations is disputed due to the lack of universal markers owing to tumor heterogeneity, it tremendously contributed to current knowledge, including that of EC [20–22] (Fig. 1).

CD44, a lymphocyte homing receptor that has a role in adhesion, motility, proliferation and cell survival [23] has extensively been studied both as a single and combined marker for CSCs. Interestingly, several CD44 variants were suggested to be a prognostic marker for adenocarcinoma of Barrett's esophagus [24] and ESCC [25]. Li et. al. [26] first suggested tumor stem-like cells to express CD44, being enriched in culture and highly expressed after irradiation. Next, Zhao et al. [27] showed increased colony formation, drug resistance and ESCC tumor initiation of CD44 cells. Regretfully, CD44 is being expressed by the majority of ESCCs in KYSE30 cells [28]. Combining CD44 with other markers greatly enhances its discriminative properties. As such, we [17] identified a $CD44^+/CD24^-$ subpopulation with CSC-like characteristics in esophageal cell lines OE33 (EAC), OE21 (ESCC), and in EC tumor biopsies. CD24, a heat-stable cell surface antigen, has a role in cell–matrix and cell–cell interactions [17,29]. $CD44^+/CD24^-$ cells had higher sphere forming potential, were more resistant to irradiation, formed tumors more aggressively, resided in hypoxic niches and the proportion of $CD44^+/CD24^-$ cells correlated with the tumor growth rate [17]. Furthermore, $CD44^+/CD24^-$ were present in half of the pretreatment biopsies of patients with residual EAC but not at all in biopsies of patients with complete pathologic response after nCRT. These results suggest that $CD44^+/CD24^-$ cells have CSC-like features and may be a target for therapy [17]. Based only on in vitro data CD44 in combination with aldehyde dehydrogenase 1 (ALDH1) was suggested to identify EC stem-like cells [30,32]. Moreover, ALDH1 expression in ESCCs was correlated with poor histological differentiation, lymph node metastasis and pathologic TNM classification [31–33], Even as a single marker, ALDH1 seems to be enriched in ESCC cell line derived tumor spheres and solely $ALDH1^{high}$ cells formed lung metastasis [34–36], had high EMT potential, were more invasive, showed increased metastic potential and were related to poor patient outcome [37,38]. Therefore, ALDH1 seems to be a good candidate CSC marker alone or in combination with CD44 [30]. Other combinations published are $CD44^+/ICAM1^+$ [39] showing all major CSC-like phenotypes, and $CD44^+/CD133^+$ that predict recurrence and prognosis of ESCC [40]. So compiling evidence indicates that CD44 in combination with other markers may enrich for EC CSCs and is of at least some prognostic value.

Another cell surface marker potentially identifying EC CSCs is ABCG2, member of group G in the ATP-binding cassette (ABC) transporter family [41]. In healthy tissue ABCG2 transporter functions as a first line defense mechanism against cytotoxic substances. In the gastrointestinal tract, including the esophagus, ABCG2 is abundantly

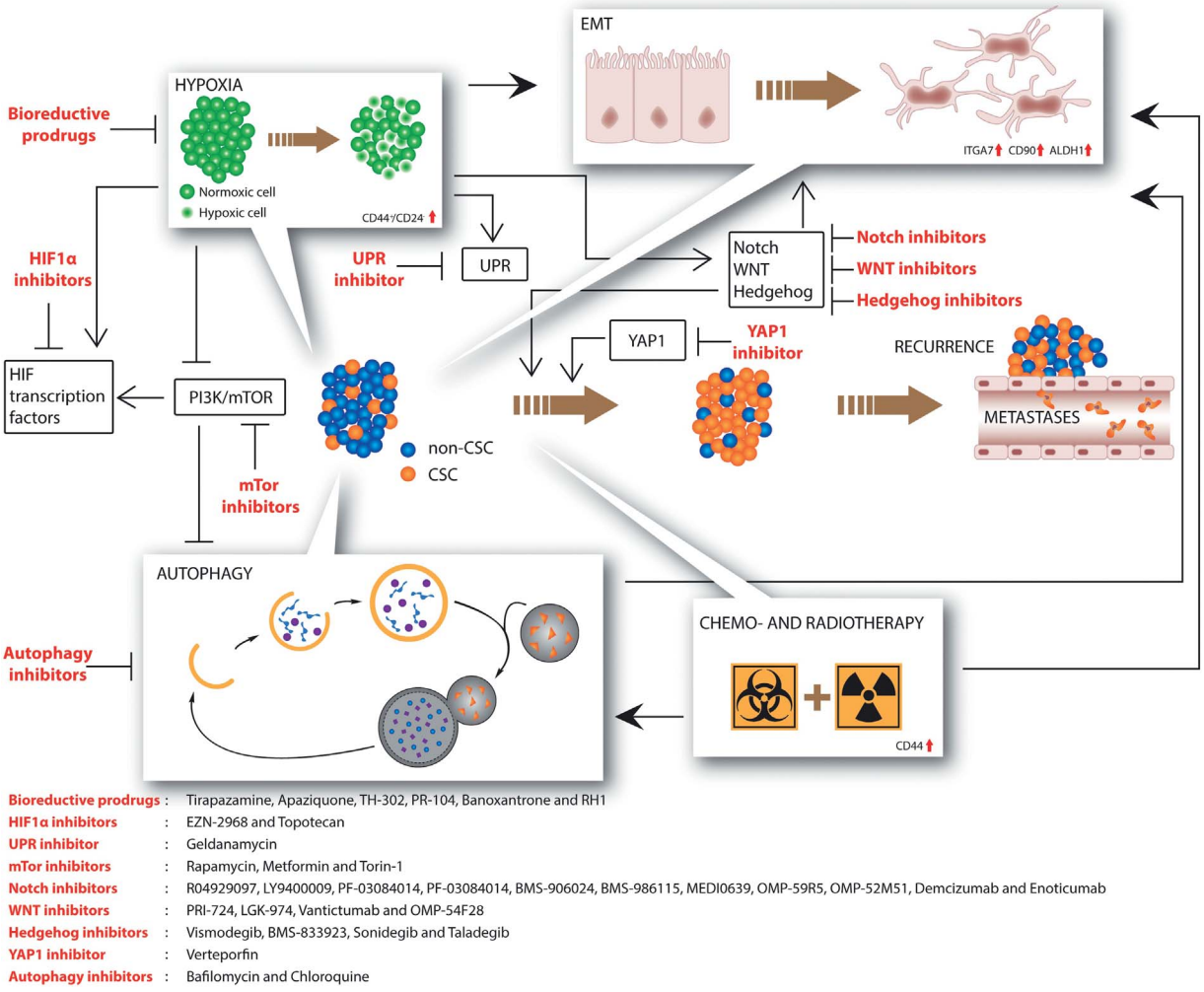

Fig. 2. The influence of the microenvironment and cellular processes in controlling the pool of esophageal CSCs. Autophagy, hypoxia, anti-cancer therapy and EMT, alone or in combination, can lead to an enrichment of CSCs through aberrant activation of pathways facilitating the development of recurrences and metastases and increasing treatment resistance. These processes can be targeted by different drugs.

expressed in the apical membrane of epithelial cells [42]. ABCG2 seems to play a role in the proliferation tumor initiation [43,44] and treatment resistance supported by reduced proliferation and migration potential of ABCG2 knockdown cells [45]. Moreover, high ABCG2 expression in ESCC surgical material correlated with the pathological tumor grading, TNM stages and with metastatic lymph nodes [43]. This suggests that ABCG2 may be an excellent marker for EC CSCs.

Additionally, CD90 or Thy-1 marked cells of EC cell lines showed increased sphere forming potential after serial passaging, efficiently generated tumors, were chemotherapy resistant, had increased invasiveness, migration and lung metastatic potential, when compared to $CD90^-$ cells, all indicative of CSC properties [46]. Integrin α7 (ITGA7), critical for modulating cell-matrix interactions, positive cells, co-express CD90 and has been suggested to mark EC CSCs with high metastatic potential [47]. Moreover, ITGA7 overexpressing cells highly expressed stemness genes (including Sox2, Oct4 and Nanog), showed EMT features, had increased self-renewal and differentiation ability and showed chemotherapy resistance. Knock down of ITGA7 reduced all these characteristics indicating that ITGA7 could be a CSC marker and a potential therapeutic target [47]. CD133 (prominin-1) has been identified as an EC CSC marker [48] and was suggested to be of prognostic value alone [49] or in combination with ABCG2 [48] or CXRC4 [50]. Finally, CD271 (p75 neurotropin receptor) expressing ESCC cells possessed CSC characeristics like self-renewal and chemotherapy resistance [51], metastatic capacity and could potential act as prognostic marker for ESCC [52]. Less usable for characterization and more general expressed in CSCs are Bmi-1, a downstream target of the Hedgehog (HH) pathway and transcription factors Sox2, Oct-4 and Nanog [53–57].

It seems clear that EC do contain cells with CSCs characteristic which express markers that might be of prognostic or predictive value. However, most data are derived from long established cell liness and tumor tissue biopsies. Patient specific organoids such as those of human metaplastic epithelia of Barrett's esophagus by Sato et al. [15] could improve our insight in CSC of EC and related stem cells markers.

3. EC CSC signaling pathways

Wnt/β-catenin, Notch, Hedgehog and Hippo pathways play an important role in proliferation, differentiation, and self-renewal of stem cells, have been implicated in the regulation of EC CSCs and are potential therapeutic targets (Fig. 1). WNT10A, an activator of the Wnt/β-catenin pathway, was highly expressed in ESCC tissue which corresponded with poor outcome. WNT10a expressing cells, showed enrichment for $CD44^+/CD24^-$ cells, increased self-renewal, invasive and metastatic potential shown by sphere forming assays and Boyden chamber assays, respectively [58]. Interestingly, Notch seems to co-drive cancer stemness in EC as inhibition of the Notch pathway using γ-secretase inhibitors downsized patient derived xenograft tumors, whereas the level of Notch activity in EC biopsies may predict response

to nCRT [59]. The Hedgehog pathway activation has been shown to be associated with distant metastases, advanced tumor stage and higher TNM stage [60–62]. Moreover, Hedgehog pathway activation may lead to stimulation of EMT [11,61,63]. The PI3K/mTOR pathway integrates a variety of signals and has been shown to interact with the Hedgehog pathway [61,64,65]. Interestingly, mTOR inhibition enhances the cytotoxic effects of Hedgehog inhibition, suggesting a rational to combine both mTOR inhibitor and Hedgehog inhibitor in future therapy [64]. Recently, the Hippo pathway, that controls organ size during development has been linked to cancer stemness EC [11,12,64,66]. Expression of YAP1, Hippo coactivator, elevated SOX9 expression accompanied by the acquisition of CSC properties, whereas knockdown of YAP1 or the use of YAP1 inhibitor Verteporfin abolished CSC phenotypes [66]. Moreover, YAP1 mediates EGFR and confers chemoresistance [67]. Interference with all these pathways using small molecules could have therapeutic benefits.

4. Epithelial-mesenchymal transition (EMT)

During cancer progression, a fraction of cancer cells may reactivate EMT, originally necessary for the dissemination of different primitive cells to various parts of the embryo [68]. Cancer cells highjack this mechanism to invade and develop metastasis [69] (Fig. 2). EMT is characterized by loss of epithelial characteristics while transforming into a multipolar, more motile and spindle-like mesenchymal phenotype [14,69–71]. Cancer cells that have undergone EMT are able to cross the endothelium, enter the blood and the lymphatic system, to regain the epithelial phenotype via a reversed process called mesenchymal-epithelial transition once the proper niche is reached and regrow [70]. EC cells that have undergone EMT through tumor microenvironment initiated activation of WNT, TGF-β and Hedgehog pathway acquired hallmarks of CSCs such as increased invasiveness, metastases and poor survival [56,63,72–78]. Radiation may induce EMT through stimulation of TGF-β1 and HIF-1α signaling increasing CD44 expression and upregulation of transcription factors such as Slug, Snail and Twist or downregulation of PTEN [79,80].

5. Hypoxia

Hypoxia is a common characteristic in locally advanced solid tumors. Poor tumor vasculature creates intratumoral hypoxic areas, inducing neovascularization as a response to oxygen and nutrition deprivation [81,82]. Hypoxia activates hypoxia-inducible factor (HIF) 1 & 2 that modulates metabolism, deregulates stem cell proliferation, enhances aggressiveness and metastatic potential [81,82] (Fig. 2), reduces radiosensitivity [83,84] induced EMT, and changes cell cycle in EC [85,86]. Interestingly, inhibition of HIF1α suppresses tumorigenicity of ESCC both in vitro and in vivo [87]. Indeed, pretreatment biopsy levels of hypoxia and HIF1α correlate with therapy resistance and poor prognosis [77,88–90]. Although not shown in EC, hypoxia targets Notch, Wnt/β-catenin, Hedgehog, PI3K/mTOR and unfolded protein response (UPR) pathways to regulate EMT and CSC stemness [91] and is activated by a number of oncogenes or loss of tumor suppressor genes [81]. Inhibition of the PI3K/mTOR pathway or a hypoxic environment leads to activation of autophagy [91] and may also be of interest in EC. mTOR pathway is a master regulator of cellular growth, proliferation, survival and metabolism and negatively regulates autophagy in response to changes in oxygen level and energy status [92].

6. Autophagy

Autophagy is an evolutionarily conserved process of eukaryotic cells designed to serve as a survival mechanism in which cell components are captured by intracellular membrane structures, degraded and recycled [91]. Upon starvation, hypoxia, pathogen invasion and chemoradiation autophagy is upregulated to maintain cellular homeostasis and to provide energy [93]. Autophagy may be a protective mechanism in the early phases of tumorigenesis requiring high levels of protein synthesis for the tumor to grow rather than protein degradation [94]. As such, inhibition of autophagy could contribute to tumor growth [94]. However, established tumors highjack autophagy to promote survival in response to cellular stress during starvation, hypoxia and therapy [94] and activate EMT, increasing invasiveness, metastatic potential and CSCs [95] (Fig. 2).

For EC it was shown that chemotherapeutic resistant cells activated autophagy which made them able to recover after therapy in contrast to chemosensitive EC cells [96]. In addition, inhibition of autophagy in resistant cell lines induced sensitization to chemotherapy [96] whereas enhancing autophagy led to higher survival in EC [97,98]. Furthermore, whereas radiation can induce autophagy and promote cell survival, combination of autophagy inhibitors with radiation enhances its deleterious effects on the tumor [99,100]. Interestingly, autophagy related markers LC3II positively and p62 negatively correlated to poor prognosis in EC patients [101–104]. Altogether, also autophagy seems to contribute greatly to the gain of EC cancer stemness by increasing drug resistance, invasiveness and the development of metastases, and may offer great potential for interference strategies.

7. The Cancer Genome Atlas (TCGA)

In the search for potential novel targets to eradicate highly metastatic CSC populations in EC, data from the TCGA network can be used. In the TCGA for gastroesophageal cancer, four subsets of genetic alterations have been identified; Epstein-Barr virus (EBV) related tumors with PIK3CA mutations/PD-L1/2 amplifications, microsatellite instability-high (MSI-H) tumors, genomic stable tumors (GS) and tumors with chromosomal instability (CIN) [105].

Moreover, in the TCGA analyses, a pattern of multiple genetic alterations has been detected with significant different molecular changes between the two main histologic types of esophageal cancer [106]. In EAC, ERBB2, VEGFA, GATA4 and GATA6 may be altered more frequently than in ESCC [105,106]. Reversely, alterations in the mTOR pathway genes PIK3CA/AKT and PTEN, TP63/SOX2 amplification and mutations in NOTCH1 are more frequently observed in ESCC [105,106]. TCGA analyses may be used in EC CSC derived organoids in both identification and validation of potentially novel biomarkers. Moreover, genome guided trials with stratification based on patient tumor derived CSC containing organoids using the TCGA distinct subsets of genetic alterations seem promising and should be developed in the near future.

8. Clinical perspectives

Although successful EC cancer therapy is measured mainly by the level of downsizing the primary tumor and minimizing metastases [107], CSCs may survive therapy and subsequently re-enter cell cycle. Therefore, future therapies should consider CSCs. Due to the anecdotal nature of the current knowledge, CSC markers have not been implemented in EC for prognosis or to monitor disease progression. Interestingly, enrichment of CSCs could result from current anti-cancer therapies due to death of bulk tumor cells and the dedifferentiation of EC cells e.g. activating EMT and autophagy [79,80,99,100]. Dedifferentiation of non-CSCs to CSCs induced by tumor microenvironment remains a huge challenge as it is not simply targeting a static population [17]. Following this notion, future anti-cancer therapy should be based on 1) eliminating the existing CSC population, by e.g. inhibitors of aberrant activated signaling pathways and 2) prevent dedifferentiation, hereby pushing the cancer cells into differentiation and making them more susceptible to conventional chemoradiation. Patient's specific aberrant activation of e.g. HH, WNT, Notch pathways, hypoxia and autophagy can be exploited to find new personalized therapeutic

targets.

8.1. HH inhibitors

Although HH inhibitors are explored extensively in clinical trials for different solid tumors, clinical trials on EC are limited. Vismodegib, a Ptch1 and SMO inhibitor regretfully did not show a survival benefit in gastroesophageal junction tumors in combination with chemotherapy (FOLFOX) [108]. Currently, Vismodegib combined with nCRT is under investigation in a clinical trial in HH activated EAC (www.NIH.com). In our hands, Vismodegib did reduce the CSC pool in EC cell lines (unpublished). Another SMO inhibitor, BMS-833923 combined with chemotherapy is currently under investigation in inoperable metastatic EC patients [109], whereas the use of SMO inhibitors sonidegib and taladegib are being explored currently [109,110].

8.2. WNT, Notch and YAP1 inhibitors

A few WNT inhibitors, PRI-724, LGK-974, Vantictumab and OMP-54F28 as a single agent or in combination with conventional therapy, are currently in clinical trial in solid cancers [109]. Unfortunately, trials on EC still need to be conducted [109]. The γ-secretase inhibitors, R04929097, LY900009, PF-03084014, BMS-906024 and BMS-986115, and MEDI0639 (anti-DLL4), OMP-59R5 (anti-Notch2/3), OMP-52M51 (anti-Notch1), Demcizumab (anti-DLL4) and Enoticumab (anti-DLL4) all inhibiting Notch signaling have made it to clinical studies in solid cancers [109]. Unfortunately, so far to our knowledge none of these drugs have been used on EC yet [109]. YAP1, the major effector target of the Hippo pathway, can also be inhibited by the small molecule Verteporfin. However, its signifance is yet to be validated in clinical trials.

8.3. Hypoxia and autophagy

Smit and colleagues showed that $CD44^+/CD24^-$ cells reside in hypoxic niches of EC xenograft derived tumors [17]. Preliminary data from our lab show increases in EC CSCs under low oxygen conditions, indicating the need to target hypoxia to eradicate all tumor cells. There are two main strategies targeting tumor hypoxia. The first one is the application of bioreductive prodrugs and the second approach is the use of inhibitors of molecular targets upon which hypoxic cells depend on [111]. A few prodrugs are currently in clinical trial such as Tirapazamine, Apaziquone, TH-302, PR-104, Banoxantrone and RH1 in other solid cancers and might provide new insights for targeting hypoxia in EC [111]. The second involve two major pathways that are essential in mediating tumor hypoxia, the HIF family of transcription factors and the UPR pathway [111]. EZN-2968 and Topotecan are targeting HIF1α while Geldanamycin is targeting the UPR pathway. mTOR inhibitors such as Metformin, Rapamycin, Torin-1 can also be explored in modulating tumor hypoxia [111]. Drugs targeting autophagy, downstream of mTOR, such as Bafilomycin and Chloroquine could also be approached in EC [110].

9. Summary

Currently, several markers to describe EC CSCs have been suggested and may be of prognostic or predictive value for response to therapy or the development of metastases. These markers as well as certain signaling pathways could be targeted in eliminating CSCs. However, the great challenge is the tumor microenvironment rendering the induction of potentially new CSC populations and needs further exploration but certainly provides avenues for drug intervention.

Although EC has a poor prognosis, targeting patients specific CSC cues may improve clinical outcome in the near future. As such, careful analysis of patient's specific tumor may lead to a personalized medicine approach, where both CSC and the bulk tumor can potentially be eradicated leading to a more satisfactory outcome for EC patients.

Conflict of interest

The authors declare that there are no conflicts of interest.

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